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Effect of cystic fibrosis exacerbations on neutrophil function

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Abstract

In cystic fibrosis (CF), inflammation is caused by persistent bacterial infection from *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* in the lung and is characterised by the persistent infiltration of massive numbers of neutrophils which leads to lung injury. The aim of this present study was to investigate the effects of CF exacerbations on the reactivity of peripheral blood neutrophils compared to data from a normal healthy control population. Peripheral blood neutrophils were isolated from control subjects and CF patients before and after an exacerbation of their lung disease. Isolated neutrophils were stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol 12-myristate 13-acetate (PMA) and the rate of superoxide generation and elastase activity measured and compared with neutrophils from healthy age-matched controls. Neutrophils from CF patients spontaneously generated higher levels of superoxide after resolution of the exacerbation compared to control neutrophils. The stimulated generation of superoxide from control neutrophils was not significantly different from neutrophils isolated from CF patients either before or after resolution of the CF exacerbation. Neutrophils from CF patients spontaneously released more elastase than control neutrophils but released less elastase than control neutrophils in response to fMLP. The stimulated release of elastase from neutrophils was not significantly different before compared to after resolution of the exacerbation. Neutrophils from CF patients displayed a different pattern of response than those from control subjects; however, CF exacerbations did not appear to modulate neutrophil function.

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1. Introduction

Cystic fibrosis (CF) is characterised by chronic progressive bronchiectasis, exocrine pancreatic insufficiency,

sterility in males, and abnormally high concentrations of sodium chloride in the sweat. Water reabsorption from the CF airway lumen outweighs limited Cl^- secretion resulting in the airway secretions becoming viscid and clearance of secretions is impaired causing airway obstruction [1]. This is further compounded by colonisation of the airway with organisms such as *Pseudomonas aeruginosa* (*P.*

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aeruginosa) and *Burkholderia cenocepacia* (*B. cenocepacia*) which are associated with a poor prognosis [2,3].

Inflammation caused by infection in the lungs plays a central role in the vicious cycle that leads to lung destruction. The most characteristic feature of inflammation in the CF lung is the persistent infiltration of massive numbers of neutrophils into the airways [4,5]. Neutrophils in the airways undergoing necrosis *in situ* are a major source of DNA, which makes CF sputum so tenacious [5]. The excessive accumulation of activated neutrophils in the lungs can lead to lung damage [6]. Proinflammatory chemoattractants have been found to be elevated in bronchoalveolar lavage fluid (BAL) from CF, including IL-8, IL-1, IL-6 and tumour necrosis factor alpha (TNF α) [7], leukotriene B₄ (LTB₄), complement factors, such as C5a, and bacterial products also act as potent chemoattractants for neutrophils [2]. Neutrophil elastase (NE) has also been shown to induce gene activation and secretion of IL-8 and digestion of C3b receptors on neutrophils, which limits the phagocytosis of pathogens [8]. In addition, levels of the anti-inflammatory cytokine IL-10 are decreased in BAL from CF patients [9]. These events in combination recruit more neutrophils to the lungs, which in turn recruit even more neutrophils setting up a perpetual inflammatory process, which ultimately causes irreparable lung damage in CF.

Neutrophils isolated from CF patients display a different pattern of response to inflammatory mediators to that observed from normals [10,11]. For example, neutrophils and eosinophils isolated from CF patients have been shown to have an altered arachidonic acid turnover [12], and an increased release of myeloperoxidase (MPO), eosinophil cationic protein (ECP), and eosinophil protein X (EPX) [13]. In addition, isolated peripheral neutrophils from CF patients generated higher levels of elastase when exposed to stimuli such as IL-8 and TNF α compared to neutrophils isolated from controls [14]. The chemotactic responsiveness of neutrophils to certain cytokines is also significantly lower in CF neutrophils compared to normal neutrophils [15–17]. Studies on bacterial products, such as *B. cepacia* lipopolysaccharide (LPS), have shown that the inflammatory nature of the *B. cepacia* infection in CF patients may contribute to increased neutrophil recruitment and priming of the neutrophil respiratory burst

[17]. This study uses *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), a compound which mimics bacterial chemotaxins and acts via a cell surface receptor, and phorbol 12-myristate 13-acetate (PMA), an analogue of diacylglycerol which activates protein kinase C directly. These compounds were chosen to investigate the receptor- and nonreceptor-activated stimulation of superoxide generation and elastase release from peripheral blood neutrophils isolated from CF patients before and after an exacerbation compared to neutrophils from normal healthy age and sex matched controls.

The hypothesis for this study was that neutrophil activity, as evidenced by the spontaneous and stimulated release of neutrophil elastase and superoxide production, would be greater in CF patients before resolution of the exacerbation. Therefore, the aim of the present study was to elucidate the effects of cystic fibrosis exacerbations on the reactivity of peripheral blood neutrophils using fMLP and PMA to investigate the rate of stimulated superoxide generation and elastase release compared to neutrophils from a normal healthy control population.

2. Methods

2.1. Subjects

Patients were recruited from those admitted to the adult CF unit of the Belfast City Hospital for a pulmonary exacerbation defined as: increased purulent sputum, decrease in FEV₁ of 10% or greater from previous best, weight loss, and decreased energy. Patients also had a documented sweat test and/or genetic analysis confirming the CF diagnosis. Patients were studied before and after resolution of the CF exacerbation. CF patients were treated with antibiotics for a minimum of 2 weeks until lung function had significantly improved and had returned to a normal level for CF. All young normal volunteers were recruited from healthy students working in the Queen's University of Belfast, who were not taking any medication. Written informed consent was obtained from all participants and the local ethics committee approved the study. As far as we are aware, none of the antibiotics used to treat the CF patients influenced neutrophil function.

2.2. Peripheral blood neutrophil isolation

Neutrophils were isolated from 50-ml whole venous blood using a previously described method [18]. Briefly, venous blood (50 ml) was collected and divided equally into two 50-ml conical centrifuge tubes, each containing 20 ml dextran (6% in 0.9% saline) and 5 ml EDTA (2.7%), and left to sediment for approximately 40 min. The resulting leukocyte-rich supernatant was removed. Carefully, 16 ml of the supernatant was laid, using a 20-ml pipette, onto 11 ml of Histopaque-1077® (density 1.077 g/ml) and centrifuged (35 min, $400\times g$, 21 °C). The plasma and Histopaque® were carefully removed leaving the neutrophils and any contaminating red blood cells (RBC) in a pellet at the bottom of the centrifuge tube. Contaminating RBC were lysed by the addition of 20 ml water (Millipore MilliQ system treated). Using a plastic Pasteur pipette, the granulocyte pellet was resuspended and then left to stand for 30 s. After 30 s, 20 ml of $2\times$ concentrated phosphate-buffered saline was added and the suspension centrifuged (10 min, $200\times g$, 21 °C). The supernatant was removed and the granulocyte pellet resuspended in 4 ml Hanks' balanced salt solution (HBSS) using a plastic Pasteur pipette. The neutrophil cell suspension was diluted with HBSS to give 4×10^6 cells/ml. Adenosine deaminase was then added (1.5 U per ml of cells) to remove endogenously produced adenosine [19]. The total cells were counted using an improved Neubauer haemocytometer. Cellular viability was studied using the Trypan blue exclusion test. Cells were >98% viable. Isolated cells were differentiated using the glass coverslip method [20]. At least 1000 cells were counted at a magnification of $\times 1000$ under oil. Cell suspensions consisted of >97% neutrophils.

2.3. Neutrophil reactive oxygen species generation using superoxide production

In this study, we measured the rate of production of superoxide anions by monitoring the reduction of cytochrome *C*, the reduced form of which has an absorption peak at 550 nm, using a Spectramax 190 plate reader (Molecular Devices, USA). This method varies slightly from the superoxide dismutase-inhibitable reduction of cytochrome *C* assay

used to quantify superoxide generation. Dose-response curves included cytochalasin b, which is used to prime the cells to produce a better response to fMLP [21]. Neutrophils were stimulated with fMLP and PMA over the concentration range 1 nM to 0.1 mM in 10-fold increments, in duplicate wells of a 96-well plate. Assay wells consisted of 50 μ l of neutrophil suspension (4×10^6 cells/ml), 120 μ l of cytochrome *C* (100 μ M), 10 μ l cytochalasin b (0.1 mg/ml), and 10 μ l of HBSS with incubation at 37 °C for 10 min. Following incubation, 10 μ l fMLP or PMA at the required concentration was added to each assay well and the change in mean optical density (mOD) recorded over the next 3 min. Blank wells contained no fMLP or PMA and an additional 10 μ l HBSS.

2.4. Neutrophil degranulation assay using elastase activity

Neutrophil degranulation was investigated by measuring the absorbance (at 405 nm) of a colorimetric elastase substrate methoxysuccinyl-alanine-alanine-proline-valine-*para*-nitroanilide (MeOSuc-Ala-Ala-Pro-Val-*p*-nitroanilide). Neutrophils were stimulated with fMLP and PMA over the concentration range 1 nM to 0.1 mM in 10-fold increments, in duplicate wells of a 96-well plate. Assay wells consisted of 50 μ l neutrophil suspension (2×10^6 cell/ml), 10 μ l cytochalasin b (0.1 mg/ml), and 100 μ l HBSS with incubation for 10 min at 37 °C. Next, 20 μ l fMLP, or PMA at the required concentration was added and incubation continued for a further 5 min. Following incubation, 20 μ l of elastase substrate (1 mg/ml) was added and the change in mean optical density (mOD) was recorded every 30 s for the next 5 min. Blank wells contained no fMLP or PMA and an additional 20 μ l HBSS.

2.5. Statistical analyses

All data are represented as mean $\pm$ S.E.M. For paired observations, Wilcoxon matched pairs test was used, and for unpaired observations, Mann-Whitney *U* test was employed. A *p* value of ≤ 0.05 was considered statistically significant. In all figures, error bars are not shown when they are smaller than the symbols used to represent that data point.

3. Results

3.1. Characterisation of the subjects

Patient and control characteristics are shown in Table 1. A total of 12 subjects was recruited in each group and the mean age of the subjects did not differ significantly. Four CF patients were colonised with *B. cenocepacia*. CF patients were treated with antibiotics for a minimum of 2 weeks until lung function returned to normal for CF. Before and after treatment, FEV₁ and FVC were significantly lower in the CF patient group compared to normal controls. As expected, FEV₁ and FVC were significantly increased after treatment in the CF patient group.

3.2. Spontaneous superoxide generation and elastase release

The rate of spontaneous superoxide generation from neutrophils isolated from CF patients before treatment was not significantly different from neutrophils isolated after treatment (Fig. 1A, $p=0.25$). There was no significant difference in the rate of superoxide generated from neutrophils from CF patients before treatment compared to neutrophils

Table 1
Characteristics of cystic fibrosis patients and control subjects

	CF patients	Controls	<i>p</i> Value
Number of subjects	12 (8 m)	12 (4 m)	–
<i>B. cenocepacia</i> positive	4 (2 m)	–	
Age (years)	24.26±1.58	27.07±1.44	0.15
Duration of treatment (days)	16.86±1.43	–	–
Mean FEV ₁ (before treatment for CF exacerbation) (L)	1.76±0.22	3.47±0.26	<0.01
Mean FEV ₁ (after treatment for CF exacerbation) (L)	2.1±0.3**	–	<0.01
Mean FVC (before treatment for CF exacerbation) (L)	2.6±0.3	4.08±0.27	<0.01
Mean FVC (after treatment for CF exacerbation) (L)	2.9±0.2*	–	0.01

FEV₁=forced expiratory volume in 1 s. FVC=forced vital capacity. Data are mean±S.E.M. CF FEV₁ was significantly higher after resolution of the exacerbation compared to before treatment (** $p=0.009$). Similarly, CF FVC was also significantly higher after resolution of the exacerbation (* $p=0.048$). The p value column refers to comparisons between CF and control characteristics. Control FEV₁ and FVC were significantly higher than CF FEV₁ and FVC before or after treatment. A p value of <0.05 was considered statistically significant.

Fig. 1. Spontaneous superoxide generation (A) and spontaneous elastase release (B) from peripheral blood neutrophils isolated from CF patients ($n=12$) before and after resolution of the exacerbation compared to control neutrophils ($n=12$). There was no statistical difference in spontaneous superoxide generation before resolution of the CF exacerbation compared to controls. However, spontaneous superoxide generation was significantly higher after resolution of the exacerbation compared to control neutrophils ($p=0.0339$). Spontaneous elastase release was significantly higher before ($p=0.0049$) and after ($p=0.0027$) resolution of the exacerbation compared to control release. There was no significant difference in superoxide generation or elastase release from CF neutrophils when comparing levels before and after resolution of the exacerbation. Data are mean±S.E.M.

from healthy controls (Fig. 1A, $p=0.20$). However, the rate of spontaneous superoxide generation from CF neutrophils after resolution of the exacerbation was significantly greater compared to neutrophils from the control group (Fig. 1A, $p=0.03$).

The rate of spontaneous elastase release from neutrophils isolated from CF patients before treatment was not significantly different from the rate of spontaneous elastase released from neutrophils isolated after treatment (Fig. 1B, $p=0.25$). However,

Fig. 2. Comparison of the effect of (A) fMLP- and (B) PMA-stimulated superoxide generation from neutrophils isolated from controls ($n=12$), and from CF patients ($n=12$) before and after treatment. Data are mean $\pm$ S.E.M. A p value of <0.05 was considered statistically significant. Error bars are not shown when they are smaller than the symbols used to represent the data points.

the rate of elastase release from neutrophils from CF patients before and after treatment was significantly higher when compared to neutrophils isolated from healthy controls (Fig. 1B, $p<0.01$ and $p<0.01$, respectively).

3.3. Superoxide generation

In all groups studied, the addition of fMLP and PMA resulted in concentration-related increases in the rate of superoxide generation from isolated peripheral neutrophils. In the control group, the addition of fMLP caused a significantly faster generation of superoxide than PMA at concentrations of 10^{-5} M ($p=0.03$), 10^{-6} M ($p=0.03$), and 10^{-7} M ($p=0.01$). There was no statistical difference in the rate of superoxide generated when neutrophils from CF

patients, before treatment, were stimulated with fMLP compared to PMA. The addition of fMLP caused a significantly faster rate of superoxide generation than PMA at concentrations of 10^{-4} M ($p=0.03$) and 10^{-7} M ($p<0.01$) from peripheral neutrophils isolated from CF patients after resolution of the exacerbation.

There were no significant differences between the rate of fMLP or PMA-induced superoxide generation between the three groups (controls, CF before and after treatment; Fig. 2A and B).

3.4. Elastase release

In all groups studied, the addition of fMLP and PMA resulted in concentration-related increases in the rate of elastase release from isolated peripheral neutrophils. PMA was a relatively poor stimulus of

Fig. 3. Comparison of the effect of (A) fMLP- and (B) PMA-stimulated elastase release from neutrophils isolated from controls ($n=12$), and CF patients ($n=12$) before and after treatment. Data are mean $\pm$ S.E.M. A p value of <0.05 was considered statistically significant. Error bars are not shown when they are smaller than the symbols used to represent the data points.

elastase release. In the control group fMLP caused a significantly higher rate of elastase release than PMA at all concentrations studied (10^{-4} M; $p<0.01$, 10^{-5} M; $p<0.01$, 10^{-6} M; $p<0.01$, 10^{-7} M; $p<0.01$, 10^{-8} M; $p=0.03$) with the exception of 10^{-9} M, which was without significant effect. FMLP induced a greater rate of elastase release than PMA at 10^{-5} , 10^{-6} , and 10^{-7} M from neutrophils isolated from CF patients before (10^{-5} M; $p=0.03$, 10^{-6} M; $p=0.03$, and 10^{-7} M; $p=0.02$) and after (10^{-5} M; $p<0.01$, 10^{-6} M; $p<0.01$, and 10^{-7} M; $p<0.01$) resolution of an exacerbation.

The addition of fMLP caused a significantly greater rate of elastase release from neutrophils isolated from control subjects compared to neutrophils from CF patients before treatment at fMLP concentrations of 10^{-4} M ($p<0.05$), 10^{-7} M ($p<0.05$), and 10^{-8} M ($p<0.01$) and from neutrophils from CF patients after treatment at all concentrations with the exception of 10^{-9} M (10^{-4} M; $p<0.05$, 10^{-5} M; $p<0.05$, 10^{-6} M; $p<0.05$, 10^{-7} M; $p<0.01$, 10^{-8} M; $p<0.001$; Fig. 3A). The rate of fMLP-stimulated elastase release from neutrophils isolated from CF patients before treatment compared to CF neutrophils after resolution of the exacerbation was similar throughout the dose–response curve (Fig. 3A). There were no significant differences in the rate of PMA-stimulated elastase release between any of the three groups (Fig. 3B).

4. Discussion

This is the first study to investigate the effects of cystic fibrosis exacerbations on peripheral blood neutrophil function. The aim of the present study was to investigate the effects of cystic fibrosis exacerbations on the reactivity of peripheral blood neutrophils using fMLP-, and PMA-stimulated superoxide generation and elastase release and compare these data to a normal healthy, age- and sex-matched control population. These stimulants were chosen because they are potent activators of neutrophil function, such as oxidative burst, degranulation, chemotaxis, and cell surface receptor up-regulation. fMLP is a bacterial tripeptide produced as a by-product of protein synthesis. It is well known that receptors for fMLP are present on the surface of neutrophils. Unlike fMLP, PMA activates protein

kinase C directly, i.e., there is no cell surface receptor for PMA. Therefore, these compounds use two very different signal transduction pathways to mediate their responses. In addition, the priming agent cytochalasin b which enhances the stimulatory effect of these stimulants by an as yet unknown mechanism [21,22] was also employed in the present study.

Studies in CF patients during exacerbations have shown increased numbers of activated neutrophils [4,23], IL-8 [2], and elastase [14] in BAL fluid. Cystic fibrosis patients also have increased oxidative stress [23] due to neutrophils [24] and consequently have been shown to have lower levels of antioxidants in the circulation [25,26]. Studies on isolated neutrophils from the peripheral blood of CF patients have shown a different response pattern to inflammatory mediators and stimulants to that seen in neutrophils from normals [10,11,27]. In this study, the spontaneous superoxide generation from neutrophils from CF patients was not significantly different after resolution of the exacerbation compared to before treatment. Similarly, there was no difference in superoxide generation from neutrophils from CF patients before treatment was commenced compared to normal controls. However, significantly more superoxide was generated from CF neutrophils after treatment compared to the normal control group, indicating that greater levels of oxidative stress may exist after treatment. The reason for this increase in spontaneous superoxide generation after treatment is hard to explain and requires further investigation. The spontaneous release of elastase from CF neutrophils poses a greater problem. Although the spontaneous elastase release was lower after resolution of the exacerbation, this did not achieve statistical significance. However, neutrophils from CF patients spontaneously released more elastase than cells isolated from healthy age-matched control subjects. This could have profound damaging effects in the lungs of CF patients. To confound the problem, lower levels of antielastase proteins, such as $\alpha 1$ -AT and SLPI, have also been demonstrated in the lungs of CF patients [28].

In the present study with control neutrophils, fMLP was a more effective stimulator of superoxide generation than PMA. In contrast, neutrophils isolated from CF patients before treatment for their exacerbations generated similar levels of superoxide in response to fMLP and PMA. Interestingly, neutrophils

isolated from CF patients after resolution of the exacerbation appeared to behave more like neutrophils from control subjects in that there was a significant difference between the fMLP and PMA response that was absent before treatment. This could suggest that there may be differences in the signal transduction pathways or receptor number in patients with CF compared to normal controls. There was no significant difference in fMLP- or PMA-induced superoxide generation from neutrophils between the three groups studied. Earlier work has demonstrated a decreased release of reactive oxygen species from CF neutrophils in response to fMLP compared to controls [27]. Studies using PMA and zymosan found that, in some CF patients, oxidative activity was higher than in controls, but in other patients, it was found to be lower than controls, indicating that there was extensive heterogeneity in oxidative activity [29].

In all groups studied, fMLP was more effective at inducing elastase release than PMA. In neutrophils isolated from CF patients either before or after treatment, PMA stimulated elastase release to the same extent. There was no difference in fMLP-induced elastase release between neutrophils isolated from CF patients before or after treatment; however, fMLP induced a faster release of elastase from control neutrophils compared to CF neutrophils. This may be due to CF neutrophils having less stored elastase due to their higher spontaneous rate of release compared to control neutrophils. Another study found that the neutrophils from CF patients released granule proteins, such as MPO, in an enhanced manner compared to normals [13], yet the data presented here suggest that CF neutrophils release less granule products than normals. The reason for this discrepancy could be that MPO could be released quickly and in greater amounts than elastase. It has also been shown that there is a decreased release of beta-glucuronidase from CF neutrophils in response to fMLP and that these differences are not due to alterations in the fMLP receptor in CF patients [27]. CF BAL has been used to stimulate peripheral neutrophils from CF patients and normals [14]. This study showed that CF neutrophils released more elastase than normal neutrophils due to TNF α and IL-8 in BAL. The combination of these cytokines could be more potent at stimulating elastase release than fMLP and PMA used individually in this study.

Overall, the data presented in this study further confirm that neutrophils from CF patients display a different pattern of response than those from control subjects. Neutrophils from CF patients spontaneously generate higher levels of superoxide after resolution of the exacerbation compared to control neutrophils. In addition, CF neutrophils spontaneously release more elastase than control neutrophils. The stimulated generation of superoxide from control neutrophils is not significantly different from neutrophils isolated from CF patients either before or after resolution of the CF exacerbation. Neutrophils isolated from CF patients release less elastase than control neutrophils in response to fMLP. The stimulated release of elastase from neutrophils was not significantly different after compared to before resolution of the exacerbation, indicating that CF exacerbations do not appear to modulate neutrophil function.

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